DETECTION OF HUMAN PAPILLOMAVIRUS E6 mRNA

The present invention is concerned with oligonucleotide primers and probes for use in detecting the presence of mRNA transcripts from the E6 gene of human papillomavirus in clinical samples.

In the last few years, there has been an improvement in the methods used to detect HPV, with methods based on amplification of nucleic acids using the polymerase chain reaction (PCR) becoming increasingly widespread. It is now possible to detect small amounts of HPV DNA (<100 pg), quantify the amount of viral DNA in clinical samples, identify a broad spectrum of genital HPV types, test for selected HPV types and localise the viral genome transcripts and proteins to the individual cells. Since HPV detection is often carried out in the presence of vast quantities of host nucleic acids and cells not infected with the virus, the ability of the primers to be virus specific is critical for a sensitive and specific amplification.

The present inventors have selected new primer and probe sequences, specific for the E6 region, which may be used in the detection of E6 transcripts by the NASBA technique, particularly sensitive, real-time NASBA, or by RT-PCR. The inventors' approach is based upon the development of primers specific for regions of E6 which are conserved across high-risk, cancer-associated HPV types.

Therefore, in accordance with a first aspect the invention provides target-specific primers and oligonucleotide probes for use in the detection of human papillomavirus (HPV) E6 mRNA, particularly for use in detection of HPV E6 mRNA by RT-PCR or NASBA.

In particular, the invention provides primer and probe oligonucleotides comprising the HPV-specific sequences represented as sequence numbers (SEQ NO) 1 to 133 in Table 1. For each individual sequence an indication is given in the column "primer/probe type" of the general types of primers or probes into which the HPV-specific sequence may be incorporated for the purposes of HPV detection. The HPV type and position in the HPV genome is also indicated.

Table 1-Summary of primer sequences

PRIMER/PROBE TYPE	SEQUENCE	SEQ NO	HPV	nt
NASBA P2/PCR	CCACAGGAGCGACCCAGAAAGTTA	1	16	116
NASBA P1/PCR	ACGGTTTGTTGTATTGCTGTTC	2	16	368
NASBA P2/PCR	CCACAGGAGCGACCCAGAAA	3	16	116
NASBA P1/PCR	GGTTTGTTGTATTGCTGTTC	4	16	368
NASBA P1/PCR	TCACGTCGCAGTAACTGT	126	16	208
NASBA P1/PCR	TTGCTTGCAGTACACACA	127	16	191
NASBA P1/PCR	TGCAGTACACACATTCTA	128	16	186
NASBA P1/PCR	GCAGTACACACATTCTAA	129	16	185
NASBA P2/PCR PROBE	ACAGTTATGCACAGAGCT	130	16	142
NASBA P2/PCR PROBE	ATATTAGAATGTGTGTAC	131	16	182
NASBA P2/PCR PROBE	TTAGAATGTGTGTACTGC	132	16	185
NASBA P2/PCR PROBE	AATGTGTGTACTGCAAG	133	16	188
PROBE	CTTTGCTTTTCGGGATTTATGC	5	16	235
PROBE	TATGACTTTGCTTTTCGGGA	6	16	230
NASBA P2/PCR	CAGAGGAGGAGGATGAAATAGTA	7	16	656
NASBA P1/PCR	GCACAACCGAAGCGTAGAGTCACAC	8	16	741
PROBE	TGGACAAGCAGAACCGGACAGAGC	9	16	687
NASBA P2/PCR	CAGAGGAGGAGGATGAAATAGA	10	16	656
NASBA P1/PCR	GCACAACCGAAGCGTAGAGTCA	11	16	741
PROBE	AGCAGAACCGGACAGAGCCCATTA	12	16	693
NASBA P2/PCR	ACGATGAAATAGATGGAGTT	13	18	702
NASBA P1/PCR	CACGGACACACAAAGGACAG	14	18	869
PROBE	AGCCGAACCACAACGTCACA	15	18	748
NASBA P2/PCR	GAAAACGATGAAATAGATGGAG	16	18	698
NASBA P1/PCR	ACACCACGGACACACAAAGGACAG	17	18	869
PROBE	GAACCACAACGTCACACAATG	18	18	752
NASBA P2/PCR	TTCCGGTTGACCTTCTATGT	19	18	651
NASBA P1/PCR	GGTCGTCTGCTGAGCTTTCT	20	18	817
NASBA P2/PCR	GCAAGACATAGAAATAACCTG	21	18	179

NASBA P1/PCR	ACCCAGTGTTAGTTAGTT	22	18	379
PROBE	TGCAAGACAGTATTGGAACT	23	18	207
NASBA P2/PCR	GGAAATACCCTACGATGAAC	24	31	164
NASBA P1/PCR	GGACACAACGGTCTTTGACA	25	31	423
PROBE	ATAGGGACGACACACCACACGGAG	26	31	268
NASBA P2/PCR	GGAAATACCCTACGATGAACTA	27	31	164
NASBA P1/PCR	CTGGACACAACGGTCTTTGACA	28	31	423
PROBE	TAGGGACGACACACCACACGGA	29	31	269
NASBA P2/PCR	ACTGACCTCCACTGTTATGA	30	31	617
NASBA P1/PCR	TATCTACTTGTGTGCTCTGT	31	31	766
PROBE	GACAAGCAGAACCGGACACATC	32	31	687
NASBA P2/PCR	TGACCTCCACTGTTATGAGCAATT	33	31	619
NASBA P1/PCR	TGCGAATATCTACTTGTGTGCTCTGT	34	31	766
PROBE	GGACAAGCAGAACCGGACACATCCAA	35	31	686
NASBA P2/PCR	ACTGACCTCCACTGTTAT	36	31	617
NASBA P1/PCR	CACGATTCCAAATGAGCCCAT	37	31	809
NASBA P2/PCR	TATCCTGAACCAACTGACCTAT	38	33	618
NASBA P1/PCR	TTGACACATAAACGAACTG	39	33	763
PROBE	CAGATGGACAAGCACAACC	40	33	694
NASBA P2/PCR	TCCTGAACCAACTGACCTAT	41	33	620
NASBA P1/PCR	CCCATAAGTAGTTGCTGTAT	42	33	807
PROBE	GGACAAGCACAACCAGCCACAGC	43	33	699
NASBA P2/PCR	GACCTTTGTGTCCTCAAGAA	44	33	431
NASBA P1/PCR	AGGTCAGTTGGTTCAGGATA	45	33	618
PROBE	AGAAACTGCACTGTGACGTGT	46	33	543
NASBA P2/PCR	ATTACAGCGGAGTGAGGTAT	47	35	217
NASBA P1/PCR	GTCTTTGCTTTTCAACTGGA	48	35	442
NASBA P2/PCR	TCAGAGGAGGAGGAAGATACTA	49	35	655
NASBA P1/PCR	GATTATGCTCTCTGTGAACA	50	35	844
NASBA P2/PCR	CCCGAGGCAACTGACCTATA	51	35	610
NASBA P1/PCR	GTCAATGTGTGTGCTCTGTA	52	35	770
PROBE	ATAGAGAAGGCCAGCCATAT	53	35	270
PROBE	GACAAGCAAAACCAGACACCTCCAA	54	35	692
PROBE	GACAAGCAAAACCAGACACC	55	35	692
NASBA P2/PCR	TTGTGTGAGGTGCTGGAAGAAT	56	52	144
NASBA P1/PCR	CCCTCTCTTCTAATGTTT	57	52	358
PROBE	GTGCCTACGCTTTTTATCTA	58	52	296
NASBA P2/PCR	GTGCCTACGCTTTTTATCTA	59	52	296
NASBA P1/PCR	GGGGTCTCCAACACTCTGAACA	60	52	507
PROBE	TGCAAACAAGCGATTTCA	61	52	461
NASBA P2/PCR	TCAGGCGTTGGAGACATC	62	58	157
NASBA P1/PCR	AGCAATCGTAAGCACACT	63	58	301
NASBA P2/PCR	TCTGTGCATGAAATCGAA	64	58	173
NASBA P1/PCR	AGCACACTTTACATACTG	65	58	291
PROBE	TGAAATGCGTTGAATGCA	66	58	192
PROBE	TTGCAGCGATCTGAGGTATATG	67	58	218
NASBA P2/PCR	TACACTGCTGGACAACAT	68	B	514
NASBA P1/PCR	TCATCTTCTGAGCTGTCT	69	B	619
NASBA P2/PCR	TACACTGCTGGACAACATGCA	70	B	514
NASBA P1/PCR	GTCACATCCACAGCAACAGGTCA	71	B	693
PROBE	GTAGGGTTACATTGCTATGA	72	B	590
PROBE	GTAGGGTTACATTGCTATGAGC	73	B	590
NASBA P2/PCR	TGACCTGTTGCTGTGGATGTGA	74	B	693

NASBA P1/PCR	TACCTGAATCGTCCGCCAT	75	B	832
PROBE	ATWGTGTGTCCCATCTGC	76	B	794
NASBA P2/PCR	CATGCCATAAATGTATAGA	77	C	295
NASBA P1/PCR	CACCGCAGGCACCTTATTAA	78	C	408
PROBE	AGAATTAGAGAATTAAGA	79	C	324
NASBA P2/PCR	GCAGACGACCACTACAGCAAA	80	39	210
NASBA P1/PCR	ACACCGAGTCCGAGTAATA	81	39	344
PROBE	ATAGGGACGGGGAACCACT	82	39	273
NASBA P2/PCR	TATTACTCGGACTCGGTGT	83	39	344
NASBA P1/PCR	CTTGGGTTTCTCTTCGTGTTA	84	39	558
PROBE	GGACCACAAAACGGGAGGAC	85	39	531
NASBA P2/PCR	GAAATAGATGAACCCGACCA	86	39	703
NASBA P1/PCR	GCACACCACGGACACACAAA	87	39	886
PROBE	TAGCCAGACGGGATGAACCACAGC	88	39	749
NASBA P2/PCR	AACCATTGAACCCAGCAGAAA	89	45	430
NASBA P1/PCR	TCTTTCTTGCCGTGCCTGGTCA	90	45	527
NASBA P2/PCR	GAAACCATTGAACCCAGCAGAAAA	91	45	428
NASBA P1/PCR	TTGCTATACTTGTGTTTCCCTACG	92	45	558
PROBE	GTACCGAGGGCAGTGTAATA	93	45	500
PROBE	GGACAAACGAAGATTTCACA	94	45	467
NASBA P2/PCR	GTTGACCTGTTGTGTTACCAGCAAT	95	45	656
NASBA P1/PCR	CACCACGGACACACAAAGGACAAG	96	45	868
NASBA P2/PCR	CTGTTGACCTGTTGTGTTACGA	97	45	654
NASBA P1/PCR	CCACGGACACACAAAGGACAAG	98	45	868
NASBA P2/PCR	GTTGACCTGTTGTGTTACGA	99	45	656
NASBA P1/PCR	ACGGACACACAAAGGACAAG	100	45	868
PROBE	GAGTCAGAGGAGGAAAACGATG	101	45	686
PROBE	AGGAAAACGATGAAGCAGATGGAGT	102	45	696
PROBE	ACAACTACCAGCCCGACGAGCCGAA	103	45	730
NASBA P2/PCR	GGAGGAGGATGAAGTAGATA	104	51	658
NASBA P1/PCR	GCCCATTAACATCTGCTGTA	105	51	807
NASBA P2/PCR	AGAGGAGGAGGATGAAGTAGATA	106	51	655
NASBA P1/PCR	ACGGGCAAACCAGGCTTAGT	107	51	829
PROBE	GCAGGTGTTCAAGTGTAGTA	108	51	747
PROBE	TGGCAGTGGAAAGCAGTGGAGACA	109	51	771
NASBA P2/PCR	TTGGGGTGCTGGAGACAAACATCT	110	56	519
NASBA P1/PCR	TTCATCCTCATCCTCATCCTCTGA	111	56	665
NASBA P2/PCR	TGGGGTGCTGGAGACAAACATC	112	56	520
NASBA P1/PCR	CATCCTCATCCTCATCCTCTGA	113	56	665
NASBA P2/PCR	TTGGGGTGCTGGAGACAAACAT	114	56	519
NASBA P1/PCR	CCACAAACTTACACTCACAACA	115	56	764
PROBE	AAAGTACCAACGCTGCAAGACGT	116	56	581
PROBE	AGAACTAACACCTCAAACAGAAAT	117	56	610
PROBE	AGTACCAACGCTGCAAGACGTT	118	56	583
PROBE	TTGGACAGCTCAGAGGATGAGG	119	56	656
NASBA P2/PCR	GATTTTCCTTATGCAGTGTG	120	56	279
NASBA P1/PCR	GACATCTGTAGCACCTTATT	121	56	410
PROBE	GACTATTCAGTGTATGGAGC	122	56	348
PROBE	CAACTGAYCTMYACTGTTATGA	123	A	
PROBE	GAAMCAACTGACCTAYWCTGCTAT	124	A	
PROBE	AAGACATTATTCAGACTC	125	A	

Oligonucleotides for use as NASBA P1 primers have the general structure "X_1-SEQ", wherein "X_1" represents a nucleotide sequence comprising a promoter and "SEQ" represents the HPV-specific sequence, as given in Table 1. The inclusion of a promoter sequence is essential in NASBA P1 primers but is not necessary in PCR primers, as discussed below. In a preferred embodiment, X_1 may be a sequence comprising a bacteriophage promoter, preferably the T7 promoter. In the most preferred embodiment, X_1 represents the sequence AATTCTAATACGACTCACTATAGGGAGAAGG.

The oligonucleotide molecules of the invention are selected to be specific for mRNA transcribed from the HPV E6 gene. Active expression of the E7 and E6 genes of HPV is associated with cervical cytological abnormalities which often progress to more serious disease. A number of studies relate the expression of the E6 and E7 genes to oncogenesis. Co-operation between E6 and E7 increases significantly the frequency of immortalization. Evidence has been presented that the E6 and E7 open reading frames are involved in the transforming activity of the virus (Tanaka et al., J. Virol. 63: 1465-1469, 1989). These transformation effects of E6 and E7 may at least in part be explained by their interaction with the cellular tumour suppressor gene products p53 and pRb 105, respectively (Boyer et al., Cancer Research. 56: 4620-4624, 1996; Lechner et al. EMBO J. 11: 3045-3051, 1992).

HPV16 mRNA isolated from transfected cells and a variety of tumour cell lines and lesions containing both extrachromosomal and integrated HPV16 genomes has

been analysed in multiple laboratories (see Doorbar JA et al., Virology 178:254?262, Rohlfs et al., Virology 183:331?342; Sherman et al., Int. J. Cancer 50:356?364). These studies have shown that several different alternatively spliced transcripts may be produced from the E6 and E7 region. In summary, there are four major transcripts: one with the whole E6/E7 gene area (E6), one with a loss of a coding sequence between basepairs 226 and 409 (E6*I), one with a loss of a coding sequence in a larger part of E6 between 226 and 526 (E6*II) and one with the loss of the E7 transcript (E6*III). However there are clearly consensus sequences in the area up to 226 basepairs in the E6 region. The inventors therefore selected the areas between 97 and 226 and between 526 and 880 as areas to target for diagnostic purposes.

The oligonucleotides provided by the invention may be grouped according to specificity for different specific HPV types or groups of HPV types. Sequence numbers 1-12 and 126-133 are specific for HPV type 16, sequence numbers 13-23 are specific for HPV type 18, sequence numbers 24-37 are specific for HPV type 31, sequence numbers 38-46 are specific for HPV type 33. HPV types 16, 18 , 31 and 33 are the major cancer-associated HPV types. Sequence numbers 47-55 are specific for HPV type 35, sequence numbers 56-61 are specific for HPV type 52, sequence numbers 62-67 are specific for HPV type 58, sequence numbers 80-88 are specific for HPV type 39, sequence numbers 89-103 are specific for HPV type 45, sequence numbers 104-109 are specific for HPV type 51, sequence numbers 110-122 are specific for HPV type 56. Sequence numbers 68-76 are consensus sequences for group B HPV types (in

particular HPV types 6 and 11). Sequence numbers 77-79 and 125 are consensus sequences for group C HPV types (including HPV types 18, 39 and 45). Sequence numbers 123 and 124 are consensus probe sequences for group A HPV types. Sequence 123 is a consensus for HPV types 16, 31 and 35; sequence 124 is a consensus for HPV types 33, 52 and 58).

The oligonucleotide molecules of the invention are preferably single stranded DNA molecules. Non-natural synthetic polynucleotides which retain the ability to base-pair with a complementary nucleic acid molecule and are also within the scope of the invention, including synthetic oligonucleotides which incorporate modified bases and synthetic oligonucleotides wherein the links between individual nucleosides include bonds other than phosphodiester bonds. The oligonucleotide molecules of the invention may be produced according to techniques well known in the art, such as by chemical synthesis using standard apparatus and protocols for oligonucleotide synthesis.

The oligonucleotide molecules provided by the invention will typically be isolated single-stranded polynucleotides of no more than 100 bases in length, more typically less than 55 bases in length. For the avoidance of doubt it is hereby stated that the language "oligonucleotide comprising sequence number n" excludes the naturally occurring full-length HPV genomes.

The invention provides several general types of oligonucleotide primers and probes incorporating the HPV-specific sequences listed in Table 1. Typically,

such oligonucleotides may comprise additional, non-HPV sequences, for example sequences which are required for an amplification reaction or which facilitate detection of the products of the amplification reaction. The HPV-specific part of the oligonucleotide may consist of one of the sequences listed in Table 1 in the absence of any other contiguous HPV sequences. However, it will be appreciated that minor variations may be made to the HPV-specific sequences, for example the addition, deletion or substitution of bases, without affecting the ability of the oligonucleotide to bind to its target sequence and function as a primer or probe to a material extent.

The first type of oligonucleotides are primer 1 oligonucleotides (also referred to herein as NASBA P1 primers), which are oligonucleotides of generally approximately 50 bases in length, containing an average of about 20 bases at the 3' end that are complementary to a region of the target mRNA. Oligonucleotides suitable for use as NASBA P1 primers are denoted "NASBA P1/PCR" in Table 1. The 5' ends of the P1 primer oligonucleotides (represented herein in general terms as X_1) comprise a promoter sequence that is recognized by a specific RNA polymerase. Bacteriophage promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the oligonucleotides of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. In a preferred embodiment, the 5' terminal sequence of the P1 primer oligonucleotides may comprise the sequence AATTCTAATACGACTCACTATAGGG or

the sequence AATTCTAATACGACTCACTATAGGGAGAAGG. These sequences contains a T7 promoter, including the transcription initiation site for T7 RNA polymerase. The HPV-specific sequences denoted in Table 1 as "NASBA P1/PCR" are suitable for use in both NASBA P1 primers and standard PCR primers. When these sequences are used as the basis of NASBA P1 primers they have the general structure X_1-SEQ, wherein X_1 represents a sequence comprising a promoter and SEQ represents the HPV-specific sequence. The promoter sequence X_1 is essential. However, when the same sequences are used as the basis of standard PCR primers it is not necessary to include X_1. The phrase "sequence number" as used in the claims is to be interpreted accordingly.

For the avoidance of doubt, the phrase "a NASBA P1 primer comprising sequence number 1" is to be interpreted as requiring the presence of an X_1 sequence 5' to the HPV-specific sequence listed as sequence number 1, whereas the phrase "a PCR primer comprising sequence number 1" refers to any suitable PCR primer comprising the HPV-specific sequence, X_1 not being an essential feature of a PCR primer. The phrase "an oligonucleotide primer including sequence number n" is taken to encompass NASBA P1, NASBA P2 and PCR primers.

A second type of oligonucleotide provided by the invention are NASBA primer 2 oligonucleotides (also referred to herein as NASBA P2 primers) which generally comprise a sequence of approximately 20 bases substantially identical to a region of the target mRNA. The oligonucleotide sequences denoted in

Table 1 as "NASBA P2/PCR" are suitable for use in both NASBA P1 primers and standard PCR primers.

Oligonucleotides intended for use as NASBA P2 primers may, in a particular but non-limiting embodiment, further comprise a sequence of nucleotides at the 5' end which is unrelated to the target mRNA but which is capable of hybridising to a generic detection probe. The detection probe will preferably be labelled, for example with a fluorescent, luminescent or enzymatic label. In one embodiment the detection probe is labelled with a label that permits detection using ECL™ technology, although it will be appreciated that the invention is in no way limited to this particular method of detection. In a preferred embodiment the 5' end of the primer 2 oligonucleotides may comprise the sequence GATGCAAGGTCGCATATGAG. This sequence is capable of hybridising to a generic ECL™ probe commercially available from Organon Teknika having the following structure:

$Ru(bpy)_3^{2+}$-GAT GCA AGG TCG CAT ATG AG-3'

In a different embodiment the primer 2 oligonucleotide may incorporate "molecular beacons" technology, which is known in the art and described, for example, in WO 95/13399 by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996, to allow for real-time monitoring of the NASBA reaction.

A third type of oligonucleotide molecules provided by the invention are target-specific probe oligonucleotides (denoted "probe" in Table 1). The probe oligonucleotides generally comprise a sequence of approximately 20-25 bases substantially identical

to a region of the target mRNA, or the complement thereof. The probe oligonucleotides may be used as target-specific hybridisation probes for detection of the products of a NASBA or PCR reaction. In this connection the probe oligonucleotides may be coupled to a solid support, such as paramagnetic beads, to form a capture probe (see below). In a preferred embodiment the 5' end of the probe oligonucleotide may be labelled with biotin. The addition of a biotin label facilitates attachment of the probe to a solid support via a biotin/streptavidin or biotin/avidin linkage.

A fourth type of oligonucleotide molecules provided by the invention are target-specific probes incorporating "molecular beacons" technology which is known in the art and described, for example, by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996 and in WO 95/13399.

The term "molecular beacons probes" as used herein is taken to mean molecules having the structure:

X_2-arm_1-target-arm_2-X_3

wherein "target" represents a target-specific sequence of nucleotides, "X_2" and "X_3" represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity and "arm_1" and "arm_2" represent complementary sequences capable of forming a stem duplex.

The invention provides molecular beacons probes incorporating a target-specific sequence comprising one of sequence numbers 6, 18, 35, 43, 123, 124 or 125.

Suitable pairs of arm_1 and arm_2 sequences for use with these HPV-specific sequences include, but not exclusively, the following:

For use with sequence number 6:

CGCATG---------CATGCG

CCAGCT---------AGCTGG

CACGC----------GCGTG

CGATCG---------CGATCG

For use with sequence number 18:

CGCATG---------CATGCG

CCGTCG---------CGACGG

CGGACC---------GGTCCG

CGATCG---------CGATCG

For use with sequence number 35:

CCGAAGG-------CCTTCGG

CCGTCG---------CGACGG

CACGTCG-------CGACGTG

CGCAGC---------GCTGCG

CGATCG---------CGATCG

For use with sequence number 43:

CCAAGC---------GCTTGG

CCAAGCG-------CGCTTGG

CCCAGC---------GCTGGG

CCAAAGC-------GCTTTGG

CCTGC----------GCAGG

CGATCG---------CGATCG

For use with sequence number 123:

CGCATG---------CATGCG

CCGTCG---------CGACGG

CCACCC----------GGGTGG

CGATCG----------CGATCG

For use with sequence number 124:

CCAAGC----------GCTTGG

CCAAGCC---------GGCTTGG

CCAAGCG---------GCGTTGG

CCAGCG----------CGCTGG

CGATCG----------CGATCG

For use with sequence number 125:

CCAAGC----------GCTTGG

CGCATG----------CATGCG

CCCAGC----------GCTGGG

CGATCG----------CGATCG

The use of probe molecules incorporating molecular beacons technology allows for real-time monitoring of amplification reactions, such as NASBA or RT-PCR reactions. The use of molecular beacons technology allows for real-time monitoring of the NASBA reaction (see Leone et al., Nucleic Acids Research., 1998, vol: 26, pp 2150-2155). The molecular beacons probes generally include complementary sequences flanking the HPV-specific sequence, represented herein by the notation arm_1 and arm_2, which are capable of hybridising to each other form a stem duplex structure. The precise sequences of arm_1 and arm_2 are not material to the invention,

except for the requirement that these sequences must be capable of forming a stem duplex when the probe is not bound to a target HPV sequence.

Molecular beacons probes also include a fluorescent moiety and a quencher moiety, the fluorescent and the quencher moieties being represented herein by the notation X_2 and X_3. As will be appreciated be the skilled reader, the fluorescer and quencher moieties are selected such that the quencher moiety is capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two moieties are in close proximity, e.g. when the probe is in the hairpin "closed" conformation in the absence of the target sequence. Upon binding to the target sequence, the fluorescent and quencher moieties are held apart such that the fluorescence of the fluorescent moiety is no longer quenched.

Many examples of suitable pairs of quencher/fluorescer moieties which may be used in accordance with the invention are known in the art (see WO 95/13399, Tyagi and Kramer, ibid). A broad range of fluorophores in many different colours made be used, including for example 5-(2'-aminoethyl)aminonaphthalene-1-sulphonic acid (EDANS), fluorescein, FAM and Texas Red (see Tyagi, Bratu and Kramer, 1998, Nature Biotechnology, 16, 49-53. The use of probes labelled with different coloured fluorophores enables "multiplex" detection of two or more different probes in a single reaction vessel. A preferred quencher is 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), a

non-fluorescent chromophore, which serves as a 'universal' quencher for a wide range of fluorophores. The fluorescer and quencher moieties may be covalently attached to the probe in either orientation, either with the fluorescer at or near the 5' end and the quencher at or near the 3' end or vice versa. Protocols for the synthesis of molecular beacon probes are known in the art. A detailed protocol for synthesis is provided in a paper entitled "Molecular Beacons: Hybridization Probes for Detection of Nucleic Acids in Homogenous Solutions" by Sanjay Tyagi et al., Department of Molecular Genetics, Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA, which is available online via the PHRI website (at www.phri.nyu.edu or www.molecular-beacons.org).

Suitable combinations of the NASBA P1 and NASBA P2 primer oligonucleotide molecules provided by the invention may be used to drive a NASBA amplification reaction. In order to drive a NASBA amplification reaction the primer 1 and primer 2 oligonucleotides must be capable of priming synthesis of a double-stranded DNA from a target region of mRNA. For this to occur the primer 1 and primer 2 oligonucleotides must comprise target-specific sequences which are complementary to regions of the sense and the antisense strand of the target mRNA, respectively.

In the first phase of the NASBA amplification cycle, the so-called "non-cyclic" phase, the primer 1 oligonucleotide anneals to a complementary sequence in the target mRNA and its 3' end is extended by the

action of an RNA-dependent DNA polymerase (e.g. reverse transcriptase) to form a first-strand cDNA synthesis. The RNA strand of the resulting RNA:DNA hybrid is then digested, e.g. by the action of RNaseH, to leave a single stranded DNA. The primer 2 oligonucleotide anneals to a complementary sequence towards the 3' end of this single stranded DNA and its 3' end is extended (by the action of reverse transcriptase), forming a double stranded DNA. RNA polymerase is then able to transcribe multiple RNA copies from the now transcriptionally active promoter sequence within the double-stranded DNA. This RNA transcript, which is antisense to the original target mRNA, can act as a template for a further round of NASBA reactions, with primer 2 annealing to the RNA and priming synthesis of the first cDNA strand and primer 1 priming synthesis of the second cDNA strand. The general principles of the NASBA reaction are well known in the art (see Compton, J. Nature. 350: 91-92).

The target-specific probe oligonucleotides described herein may also be attached to a solid support, such as magnetic microbeads, and used as "capture probes" to immobilise the product of the NASBA amplification reaction (a single stranded RNA). The target-specific "molecular beacons" probes described herein may be used for real-time monitoring of the NASBA reaction.

In a particular embodiment the invention provides the oligonucleotide listed in Table 2, these being NASBA P1 primers and NASBA P2 primers containing the sequences listed in Table 1. The NASBA P1 primers further include a T7 promoter sequence, the NASBA P2

primers include a sequence for binding of a generic detection probe (see below) and associated probe molecules for use in the detection of HPV mRNA by NASBA. The oligonucleotides listed in Table 2 are merely illustrative and it is not intended that the scope of the invention should be limited to these specific molecules.

The NASBA P2 primers (p2)in Table 2 include the sequence GATGCAAGGTCGCATATGAG at the 5' end; the NASBA P1 primers (p1) in Table 2 include the sequence AATTCTAATACGACTCACTATAGGGAGAAGG at the 5' end. Oligonucleotides suitable for use as probes are identified by "po". The P2 primers generally contain HPV sequences from the postive strand, whereas the p1 primers generally contain HPV sequences from the negative strand. nt-refers to nucleotide position in the relevant HPV genomic sequence.

Table 2-NASBA primer and probe sequences

Primer name	**Sequence**	HPV Type	nt
HAe6701p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCC AGAAAGTTA	16	116
HAe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGG TTTGTTGTATTGCTGTTC	16	368
HAe6702p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCC AGAAA	16	116
HAe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTT TGTTGTATTGCTGTTC	16	368
HAe6702Ap1	AATTCTAATACGACTCACTATAGGGAGAAGGTCA CGTCGCAGTAACTGT	16	208
HAe6702Bp1	AATTCTAATACGACTCACTATAGGGAGAAGGTTG CTTGCAGTACACACA	16	191
HAe6702Cp1	AATTCTAATACGACTCACTATAGGGAGAAGGTGC AGTACACACATTCTA	16	186
HAe6702Dp1	AATTCTAATACGACTCACTATAGGGAGAAGGGCA GTACACACATTCTAA	16	185
H16e6702Ap2	GATGCAAGGTCGCATATGAGACAGTTATGCACAGA GCT	16	142

Primer name	Sequence	HPV Type	nt
H16e6702Bp2	GATGCAAGGTCGCATATGAGATATTAGAATGTGTG TAC	16	182
H16e6702Cp2	GATGCAAGGTCGCATATGAGTTAGAATGTGTGTAC TGC	16	185
H16e6702Dp2	GATGCAAGGTCGCATATGAGGAATGTGTGTACTGC AAG	16	188
H16e6702Apo	ACAGTTATGCACAGAGCT	16	142
H16e6702Bpo	ATATTAGAATGTGTGTAC	16	182
H16e6702Cpo	TTAGAATGTGTGTACTGC	16	185
H16e6702Dpo	GAATGTGTGTACTGCAAG	16	188
HAe6701po	CTTTGCTTTTCGGGATTTATGC	16	235
HAe6702po	TATGACTTTGCTTTTCGGGA	16	230
HAe6702mb1	X_2-cgcatgTATGACTTTGCTTTTCGGGAcatgcg -X_3	16	230
HAe6702mb2	X_2-ccagcttATGACTTTGCTTTTCGGGAagctgg -X_3	16	230
HAe6702mb3	X_2-cacgcTATGACTTTGCTTTTCGGGAgcgtg-X_3	16	230
H16e6702mb4	X_2-cgatcgTATGACTTTGCTTTTCGGGAcgatcg -X_3	16	230
HAe6703p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATG AAATAGTA	16	656
HAe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCAC AACCGAAGCGTAGAGTCACAC	16	741
HAe6703po	TGGACAAGCAGAACCGGACAGAGC	16	687
HAe6704p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATG AAATAGA	16	656
HAe6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCAC AACCGAAGCGTAGAGTCA	16	741
HAe6704po	AGCAGAACCGGACAGAGCCCATTA	16	693
H18e6701p2	GATGCAAGGTCGCATATGAGACGATGAAATAGATG GAGTT	18	702
H18e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACG GACACACAAAGGACAG	18	869
H18e6701po	AGCCGAACCACAACGTCACA	18	748
H18e6702p2	GATGCAAGGTCGCATATGAGGAAAACGATGAAATA GATGGAG	18	698
H18e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACAC CACGGACACACAAAGGACAG	18	869
H18e6702po	GAACCACAACGTCACACAATG	18	752
H18e6702mb1	X_2-cgcatgGAACCACAACGTCACACAATGcatgcg -X_3	18	752
H18e6702mb2	X_2-ccgtcgGAACCACAACGTCACACAATGcgacgg -X_3	18	752
H18e6702mb3	X_2-cggaccGAACCACAACGTCACACAATGggtccg -X_3	18	752
H18e6702mb4	X_2-cgatcgGAACCACAACGTCACACAATGcgatcg	18	752

Primer name	Sequence	HPV Type	nt
	$-X_3$		
H18e6703p2	GATGCAAGGTCGCATATGAGTTCCGGTTGACCTTC TATGT	18	651
H18e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTC GTCTGCTGAGCTTTCT	18	817
H18e6704p2	GATGCAAGGTCGCATATGAGGCAAGACATAGAAAT AACCTG	18	179
H18e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGACCC AGTGTTAGTTAGTT	18	379
H18e6704po	TGCAAGACAGTATTGGAACT	18	207
H31e6701p2	GATGCAAGGTCGCATATGAGGGAAATACCCTACGA TGAAC	31	164
H31e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGAC ACAACGGTCTTTGACA	31	423
H31e6701po	ATAGGGACGACACACCACACGGAG	31	268
H31e6702p2	GATGCAAGGTCGCATATGAGGGAAATACCCTACGA TGAACTA	31	164
H31e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCTGG ACACAACGGTCTTTGACA	31	423
H31e6702po	TAGGGACGACACACCACACGGA	31	269
H31e6703p2	GATGCAAGGTCGCATATGAGACTGACCTCCACTGT TATGA	31	617
H31e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGTATC TACTTGTGTGCTCTGT	31	766
H31e6703po	GACAAGCAGAACCGGACACATC	31	687
H31e6704p2	GATGCAAGGTCGCATATGAGTGACCTCCACTGTTA TGAGCAATT	31	619
H31e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGTGCG AATATCTACTTGTGTGCTCT GT	31	766
H31e6704po	GGACAAGCAGAACCGGACACATCCAA	31	686
H31e6704mb1	X_2-ccgaaggGGACAAGCAGAACCGGACACATCC AAccttcgg $-X_3$	31	686
H31e6704mb2	X_2-ccgtcgGGACAAGCAGAACCGGACACATCCA Acgacgg $-X_3$	31	686
H31e6704mb3	X_2- cacgtcgGGACAAGCAGAACCGGACACATCCAA cgacgtg $-X_3$	31	686
H31e6704mb4	X_2-cgcagcGGACAAGCAGAACCGGACACATCCAA gctgcg $-X_3$	31	686
H31e6704mb5	X_2-cgatcgGGACAAGCAGAACCGGACACATCCAA cgatcg $-X_3$	31	686
H31e6705p2	GATGCAAGGTCGCATATGAGACTGACCTCCACTGT TAT	31	617
H31e6705p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACG ATTCCAAATGAGCCCAT	31	809
H33e6701p2	GATGCAAGGTCGCATATGAGTATCCTGAACCAACT GACCTAT	33	618

Primer name	Sequence	HPV Type	nt
H33e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTGA CACATAAACGAACTG	33	763
H33e6701po	CAGATGGACAAGCACAACC	33	694
H33e6703p2	GATGCAAGGTCGCATATGAGTCCTGAACCAACTGA CCTAT	33	620
H33e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCCA TAAGTAGTTGCTGTAT	33	807
H33e6703po	GGACAAGCACAACCAGCCACAGC	33	699
H33e6703mb1	X_2-ccaagcGGACAAGCACAACCAGCCACAGCgct tgg -X_3	33	699
H33e6703mb2	X_2-ccaagcgGGACAAGCACAACCAGCCACAGC cgcttgg -X_3	33	699
H33e6703mb3	X_2-cccagcGGACAAGCACAACCAGCCACAGCgct ggg -X_3	33	699
H33e6703mb4	X_2-ccaaagcGGACAAGCACAACCAGCCACAGCg ctttgg -X_3	33	699
H33e6703mb5	X_2-cctgcGGACAAGCACAACCAGCCACAGCgcagg -X_3	33	699
H33e6703mb6	X_2-cgatcgGGACAAGCACAACCAGCCACAGCcga tcg -X_3	33	699
H33e6702p2	GATGCAAGGTCGCATATGAGGACCTTTGTGTCCTC AAGAA	33	431
H33e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGGT CAGTTGGTTCAGGATA	33	618
H33e6702po	AGAAACTGCACTGTGACGTGT	33	543
H35e6701p2	GATGCAAGGTCGCATATGAGATTACAGCGGAGTGA GGTAT	35	217
H35e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCT TTGCTTTTCAACTGGA	35	442
H35e5601po	ATAGAGAAGGCCAGCCATAT	35	270
H35e6702p2	GATGCAAGGTCGCATATGAGTCAGAGGAGGAGGAA GATACTA	35	655
H35e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGATT ATGCTCTCTGTGAACA	35	844
H35e6703p2	GATGCAAGGTCGCATATGAGCCCGAGGCAACTGAC CTATA	35	610
H35e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCA ATGTGTGTGCTCTGTA	35	770
H35e6702po	GACAAGCAAAACCAGACACCTCCAA	35	692
H35e6703po	GACAAGCAAAACCAGACACC	35	692
H52e6701p2	GATGCAAGGTCGCATATGAGTTGTGTGAGGTGCTG GAAGAAT	52	144
H52e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCCT CTCTTCTAATGTTT	52	358
H52e6701po	GTGCCTACGCTTTTTATCTA	52	296
H52e6702p2	GATGCAAGGTCGCATATGAGGTGCCTACGCTTTTT	52	296

Primer name	Sequence	HPV Type	nt
	ATCTA		
H52e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGGG TCTCCAACACTCTGAACA	52	507
H52e6702po	TGCAAACAAGCGATTTCA	52	461
H58e6701p2	GATGCAAGGTCGCATATGAGTCAGGCGTTGGAGAC ATC	58	157
H58e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCA ATCGTAAGCACACT	58	301
H58e6702p2	GATGCAAGGTCGCATATGAGTCTGTGCATGAAATC GAA	58	173
H58e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCA CACTTTACATACTG	58	291
H58e6701po	TGAAATGCGTTGAATGCA	58	192
H58e6702po	TTGCAGCGATCTGAGGTATATG	58	218
HBe6701p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAA CAT	B(11)	514
HBe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCAT CTTCTGAGCTGTCT	B(11)	619
HBe6702p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAA CATGCA	B(11)	514
HBe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCA CATCCACAGCAACAGGTCA	B(11)	693
HBe6701po	GTAGGGTTACATTGCTATGA	B(11)	590
HBe6702po	GTAGGGTTACATTGCTATGAGC	B(11)	590
HBe6703p2	GATGCAAGGTCGCATATGAGTGACCTGTTGCTGTG GATGTGA	B(11)	693
HBe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGTACC TGAATCGTCCGCCAT	B(11)	832
HBe6703po	ATWGTGTGTCCCATCTGC	B(11)	794
HCe6701p2	GATGCAAGGTCGCATATGAGCATGCCATAAATGTA TAGA	C(18 39 45)	295
HCe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACC GCAGGCACCTTATTAA	C(18 39 45	408
HCe6701po	AGAATTAGAGAATTAAGA	C(18 39 45	324
H39e6701p2	GATGCAAGGTCGCATATGAGGCAGACGACCACTAC AGCAAA	39	210
H39e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACAC CGAGTCCGAGTAATA	39	344
H39e6701po	ATAGGGACGGGGAACCACT	39	273
H39e6702p2	GATGCAAGGTCGCATATGAGTATTACTCGGACTCG GTGT	39	344
H39e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCTTG GGTTTCTCTTCGTGTTA	39	558
H39e6702po	GGACCACAAAACGGGAGGAC	39	531
H39e6703p2	GATGCAAGGTCGCATATGAGGAAATAGATGAACCC GACCA	39	703

Primer name	Sequence	HPV Type	nt
H39e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCAC ACCACGGACACACAAA	39	886
H39e6703po	TAGCCAGACGGGATGAACCACAGC	39	749
H45e6701p2	GATGCAAGGTCGCATATGAGAACCATTGAACCCAG CAGAAA	45	430
H45e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCTT TCTTGCCGTGCCTGGTCA	45	527
H45e6702p2	GATGCAAGGTCGCATATGAGGAAACCATTGAACCC AGCAGAAAA	45	428
H45e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTGC TATACTTGTGTTTCCCTACG	45	558
H45e6701po	GTACCGAGGGCAGTGTAATA	45	500
H45e6702po	GGACAAACGAAGATTTCACA	45	467
H45e6703p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGT TACCAGCAAT	45	656
H45e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACC ACGGACACACAAAGGACAAG	45	868
H45e6704p2	GATGCAAGGTCGCATATGAGCTGTTGACCTGTTGT GTTACGA	45	654
H45e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCAC GGACACACAAAGGACAAG	45	868
H45e6705p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGT TACGA	45	656
H45e6705p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGG ACACACAAAGGACAAG	45	868
H45e6703po	GAGTCAGAGGAGGAAAACGATG	45	686
H45e6704po	AGGAAAACGATGAAGCAGATGGAGT	45	696
H45e6705po	ACAACTACCAGCCCGACGAGCCGAA	45	730
H51e6701p2	GATGCAAGGTCGCATATGAGGGAGGAGGATGAAGT AGATA	51	658
H51e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCCC ATTAACATCTGCTGTA	51	807
H51e6702p2	GATGCAAGGTCGCATATGAGAGAGGAGGAGGATGA AGTAGATA	51	655
H51e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGG GCAAACCAGGCTTAGT	51	829
H51e6701po	GCAGGTGTTCAAGTGTAGTA	51	747
H51e6702po	TGGCAGTGGAAAGCAGTGGAGACA	51	771
H56e6701p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGA CAAACATCT	56	519
H56e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTCA TCCTCATCCTCATCCTCTGA	56	665
H56e6702p2	GATGCAAGGTCGCATATGAGTGGGGTGCTGGAGAC AAACATC	56	520
H56e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCATC CTCATCCTCATCCTCTGA	56	665
H56e6703p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGA	56	519

Primer name	Sequence	HPV Type	nt
	CAAACAT		
H56e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCAC AAACTTACACTCACAACA	56	764
H56e6701po	AAAGTACCAACGCTGCAAGACGT	56	581
H56e6702po	AGAACTAACACCTCAAACAGAAAT	56	610
H56e6703po	AGTACCAACGCTGCAAGACGTT	56	583
H56e6703po1	TTGGACAGCTCAGAGGATGAGG	56	656
H56e6704p2	GATGCAAGGTCGCATATGAGGATTTTCCTTATGCA GTGTG	56	279
H56e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGACA TCTGTAGCACCTTATT	56	410
H56e6704po	GACTATTCAGTGTATGGAGC	56	348
HPVAPO1A	CAACTGAYCTMYACTGTTATGA	A (16 31 35)	
HPVApo1Amb1	X_2-cgcatgCAACTGAYCTMYACTGTTATGAcatgcg -X_3	A (16 31 35)	
HPVApo1Amb2	X_2-ccgtcgCAACTGAYCTMYACTGTTATGAcga cgg -X_3	A (16 31 35)	
HPVApo1Amb3	X_2-ccacccCAACTGAYCTMYACTGTTATGAgg gtgg -X_3	A (16 31 35)	
HPVApo1Amb4	X_2-cgatcgCAACTGAYCTMYACTGTTATGAcga tcg -X_3	A (16 31 35)	
HPVAPO4A	GAAMCAACTGACCTAYWCTGCTAT	A (33 52 58)	
HPVAPO4Amb1	X_2-ccaagcGAAMCAACTGACCTAYWCTGCTATgc ttgg -X_3	A (33 52 58)	
HPVAPO4Amb2	X_2-ccaagccGAAMCAACTGACCTAYWCTGCTAT ggcttgg -X_3	A (33 52 58)	
HPVAPO4Amb3	X_2-ccaagcgGAAMCAACTGACCTAYWCTGCTA Tcgcttgg -X_3	A (33 52 58)	
HPVAPO4Amb4	X_2-ccagcgGAAMCAACTGACCTAYWCTGCTATcg ctgg -X_3	A (33 52 58)	
HPVAPO4Amb5	X_2-cgatcgGAAMCAACTGACCTAYWCTGCTATcg atcg -X_3	A (33 52 58)	
HPVCPO4	AAGACATTATTCAGACTC	C (18 45 39)	
HPVCPO4Amb1	X_2-ccaagcAAGACATTATTCAGACTCgcttgg -X_3	C (18 45 39)	
HPVCPO4Amb2	X_2-cgcatgAAGACATTATTCAGACTCcatgcg -X_3	C (18 45 39)	
HPVCPO4Amb3	X_2-cccagcAAGACATTATTCAGACTCgctggg -X_3	C (18 45 39)	
HPVCPO4Amb4	X_2-cgatcgAAGACATTATTCAGACTCcgatcg -X_3	C (18 45 39)	

The meaning of X_2- and -X_3 is defined above, in the discussion of "molecular beacons" probe molecules.

In a further embodiment the invention provides the oligonucleotides listed in Table 3, these being PCR primers for use in the detection of HPV mRNA by RT-PCR. These oligonucleotides are merely illustrative and it is not intended that the scope of the invention should be limited to these specific molecules:

Primer name	Sequence	HPV type	nt
HAe6701PCR2	CCACAGGAGCGACCCAGAAAGTTA	16	116
HAe6701PCR1	ACGGTTTGTTGTATTGCTGTTC	16	368
HAe6702PCR2	CCACAGGAGCGACCCAGAAA	16	116
HAe6702PCR1	GGTTTGTTGTATTGCTGTTC	16	368
HAe6703PCR2	CAGAGGAGGAGGATGAAATAGTA	16	656
HAe6703PCR1	GCACAACCGAAGCGTAGAGTCACAC	16	741
HAe6704PCR2	CAGAGGAGGAGGATGAAATAGA	16	656
HAe6704PCR1	GCACAACCGAAGCGTAGAGTCA	16	741
H18e6701PCR2	ACGATGAAATAGATGGAGTT	18	702
H18e6701PCR1	CACGGACACACAAAGGACAG	18	869
H18e6702PCR2	GAAAACGATGAAATAGATGGAG	18	698
H18e6702PCR1	ACACCACGGACACACAAAGGACAG	18	869
H18e6703PCR2	TTCCGGTTGACCTTCTATGT	18	651
H18e6703PCR1	GGTCGTCTGCTGAGCTTTCT	18	817
H18e6704PCR2	GCAAGACATAGAAATAACCTG	18	179
H18e6704PCR1	ACCCAGTGTTAGTTAGTT	18	379
H31e6701PCR2	GGAAATACCCTACGATGAAC	31	164
H31e6701PCR1	GGACACAACGGTCTTTGACA	31	423
H31e6702PCR2	GGAAATACCCTACGATGAACTA	31	164
H31e6702PCR1	CTGGACACAACGGTCTTTGACA	31	423
H31e6703PCR2	ACTGACCTCCACTGTTATGA	31	617
H31e6703PCR1	TATCTACTTGTGTGCTCTGT	31	766
H31e6704PCR2	TGACCTCCACTGTTATGAGCAATT	31	619
H31e6704PCR1	TGCGAATATCTACTTGTGTGCTCT GT	31	766
H31e6705PCR2	ACTGACCTCCACTGTTAT	31	617
H31e6705PCR1	CACGATTCCAAATGAGCCCAT	31	809
H33e6701PCR2	TATCCTGAACCAACTGACCTAT	33	618
H33e6701PCR1	TTGACACATAAACGAACTG	33	763
H33e6703PCR2	TCCTGAACCAACTGACCTAT	33	620
H33e6703PCR1	CCCATAAGTAGTTGCTGTAT	33	807
H33e6702PCR2	GACCTTTGTGTCCTCAAGAA	33	431
H33e6702PCR1	AGGTCAGTTGGTTCAGGATA	33	618
H35e6701PCR2	ATTACAGCGGAGTGAGGTAT	35	217
H35e6701PCR1	GTCTTTGCTTTTCAACTGGA	35	442
H35e6702PCR2	TCAGAGGAGGAGGAAGATACTA	35	655

Primer name	Sequence	HPV type	nt
H35e6702PCR1	GATTATGCTCTCTGTGAACA	35	844
H35e6703PCR2	CCCGAGGCAACTGACCTATA	35	610
H35e6703PCR1	GTCAATGTGTGTGCTCTGTA	35	770
H52e6701PCR2	TTGTGTGAGGTGCTGGAAGAAT	52	144
H52e6701PCR1	CCCTCTCTTCTAATGTTT	52	358
H52e6702PCR2	GTGCCTACGCTTTTTATCTA	52	296
H52e6702PCR1	GGGGTCTCCAACACTCTGAACA	52	507
H58e6701PCR2	TCAGGCGTTGGAGACATC	58	157
H58e6701PCR1	AGCAATCGTAAGCACACT	58	301
H58e6702PCR2	TCTGTGCATGAAATCGAA	58	173
H58e6702PCR1	AGCACACTTTACATACTG	58	291
HBe6701PCR2	TACACTGCTGGACAACAT	B(11)	514
HBe6701PCR1	TCATCTTCTGAGCTGTCT	B(11)	619
HBe6702PCR2	TACACTGCTGGACAACATGCA	B(11)	514
HBe6702PCR1	GTCACATCCACAGCAACAGGTCA	B(11)	693
HBe6703PCR2	TGACCTGTTGCTGTGGATGTGA	B(11)	693
HBe6703PCR1	TACCTGAATCGTCCGCCAT	B(11)	832
HCe6701PCR2	CATGCCATAAATGTATAGA	C (18 39 45	295
HCe6701PCR1	CACCGCAGGCACCTTATTAA	C (18 39 45	408
H39e6701PCR2	GCAGACGACCACTACAGCAAA	39	210
H39e6701PCR1	ACACCGAGTCCGAGTAATA	39	344
H39e6702PCR2	TATTACTCGGACTCGGTGT	39	344
H39e6702PCR1	CTTGGGTTTCTCTTCGTGTTA	39	558
H39e6703PCR2	GAAATAGATGAACCCGACCA	39	703
H39e6703PCR1	GCACACCACGGACACACAAA	39	886
H45e6701PCR2	AACCATTGAACCCAGCAGAAA	45	430
H45e6701PCR1	TCTTTCTTGCCGTGCCTGGTCA	45	527
H45e6702PCR2	GAAACCATTGAACCCAGCAGAAAA	45	428
H45e6702PCR1	TTGCTATACTTGTGTTTCCCTACG	45	558
H45e6703PCR2	GTTGACCTGTTGTGTTACCAGCAAT	45	656
H45e6703PCR1	CACCACGGACACACAAAGGACAAG	45	868
H45e6704PCR2	CTGTTGACCTGTTGTGTTACGA	45	654
H45e6704PCR1	CCACGGACACACAAAGGACAAG	45	868
H45e6705PCR2	GTTGACCTGTTGTGTTACGA	45	656
H45e6705PCR1	ACGGACACACAAAGGACAAG	45	868
H51e6701PCR2	GGAGGAGGATGAAGTAGATA	51	658
H51e6701PCR1	GCCCATTAACATCTGCTGTA	51	807
H51e6702PCR2	AGAGGAGGAGGATGAAGTAGATA	51	655
H51e6702PCR1	ACGGGCAAACCAGGCTTAGT	51	829
H56e6701PCR2	TTGGGGTGCTGGAGACAAACATCT	56	519
H56e6701PCR1	TTCATCCTCATCCTCATCCTCTGA	56	665
H56e6702PCR2	TGGGGTGCTGGAGACAAACATC	56	520
H56e6702PCR1	CATCCTCATCCTCATCCTCTGA	56	665
H56e6703PCR2	TTGGGGTGCTGGAGACAAACAT	56	519
H56e6703PCR1	CCACAAACTTACACTCACAACA	56	764
H56e6704PCR2	GATTTTCCTTATGCAGTGTG	56	279
H56e6704PCR1	GACATCTGTAGCACCTTATT	56	410

Primer-pairs and primer-probe sets

The invention further provides primer-pairs and primer/probe sets for use in the detection of HPV E6 transcripts.

A "primer-pair" is taken to mean two primers which may be used in combination for amplification of a portion of an HPV E6 transcript, for example by NASBA or RT-PCR. The individual oligonucleotide primers making up the primer-pair may be supplied separately, e.g. in separate containers. A primer-pair may also be supplied as a homogenous mixture of the two primers, this mixture may include additional reagents required for the amplification reaction, as discussed below.

A "primer/probe set" is taken to mean a set of oligonucleotides comprising a primer-pair, as defined above, and at least one oligonucleotide probe which is suitable for use in detection of an amplification product generated by use of the primer-pair. The individual oligonucleotides making up the primer/probe set may be supplied separately, e.g. in separate containers or as a homogenous mixture.

In this context "primer" is taken to encompass primers suitable for use in PCR and primers suitable for use in NASBA.

The term "probe" may encompass any of the probe types described herein, including molecular beacons probes suitable for use in real-time NASBA (see below) and capture probes for immobilisation of NASBA amplification products.

Specific primer-pairs provided by the invention are given below, together with suitable probes which may be used in the detection of amplification products

generated using the primer-pair. In preferred embodiments, the primer-pairs listed below may comprise a NASBA P1 primer and a NASBA P2 primer or two PCR primers. The most preferred specific primer combinations are listed, using the primer names given in Tables 2 and 3. However, it is not intended to limit the scope of the invention to these particular combinations:

Primer-pairs and probes for use in the detection of mRNA transcripts from the E6 gene of HPV 16:

(1) an oligonucleotide primer comprising sequence number 1 and an oligonucleotide primer comprising sequence number 2; oligonucleotide probe comprising sequence number 5.

Preferred NASBA primers: HAe6701p1 and HAe6701p2
Preferred PCR primers: HAe6701PCR1 and HAe6701PCR2

(2) an oligonucleotide primer comprising sequence number 3 and an oligonucleotide primer comprising sequence number 4; oligonucleotide probe comprising sequence number 6.

Preferred NASBA primers: HAe6702p1 and HAe6702p2
Preferred PCR primers: HAe 6702PCR1 and HAe6702PCR2

(3) an oligonucleotide primer comprising sequence number 7 and an oligonucleotide primer comprising sequence number 8; oligonucleotide probe comprising sequence number 9.

Preferred NASBA primers: HAe6703p1 and HAe6703p2
Preferred PCR primers: HAe6703PCR1 and HAe6703PCR2

(4) an oligonucleotide primer comprising sequence number 10 and an oligonucleotide primer comprising sequence number 11; oligonucleotide probe comprising sequence number 12.

Preferred NASBA primers: HAe6704p1 and HAe6704p2
Preferred PCR primers: HAe6704PCR1 and HAe6704PCR2

(5) an oligonucleotide primer comprising one of sequence numbers 126, 127, 128 or 129 and an oligonucleotide primer comprising sequence number 1 or sequence number 3.

(6) an oligonucleotide primer comprising sequence number 2 or sequence number 4 and an oligonucleotide primer comprising one of sequence numbers 130, 131, 132 or 133.

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 18:

(7) an oligonucleotide primer comprising sequence number 13 and an oligonucleotide primer comprising sequence number 14; oligonucleotide probe comprising sequence number 15.

Preferred NASBA primers: H18e6701p1 and H18e6701p2
Preferred PCR primers: H18e6701PCR1 and H18e6701PCR2

(8) an oligonucleotide primer comprising sequence number 16 and an oligonucleotide primer comprising sequence number 17; oligonucleotide probe comprising sequence number 18.

Preferred NASBA primers: H18e6702p1 and H18e6702p2
Preferred PCR primers: H18e6702PCR1 and H18e6702PCR2

(9) an oligonucleotide primer comprising sequence number 19 and an oligonucleotide primer comprising sequence number 20.

Preferred NASBA primers: H18e6703p1 and H18e6703p2
Preferred PCR primers: H1836703PCR1 and H18e6703PCR2

(10) an oligonucleotide primer comprising sequence number 21 and an oligonucleotide primer comprising sequence number 22; oligonucleotide probe comprising sequence number 23.

Preferred NASBA primers: H18e6704p1 and H18e6704p2
Preferred PCR primers: H18e6704PCR1 and H18e6704PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 31:

(11) an oligonucleotide primer comprising sequence number 24 and an oligonucleotide primer comprising sequence number 25; oligonucleotide probe comprising sequence number 26.

Preferred NASBA primers: H31e6701p1 and H31e6701p2
Preferred PCR primers: H31e6701PCR1 and H31e6701PCR2

(12) an oligonucleotide primer comprising sequence number 27 and an oligonucleotide primer comprising sequence number 28; oligonucleotide probe comprising sequence number 29.

Preferred NASBA primers: H31e6702p1 and H31e6702p2
Preferred PCR primers: H31e6702PCR1 and H3136702PCR2

(13) an oligonucleotide primer comprising sequence number 30 and an oligonucleotide primer comprising

sequence number 31; oligonucleotide probe comprising sequence number 32.

Preferred NASBA primers: H31e6703p1 and H31e6703p2
Preferred PCR primers: H31e6703PCR1 and H31e6703PCR2

(14) an oligonucleotide primer comprising sequence number 33 and an oligonucleotide primer comprising sequence number 34; oligonucleotide probe comprising sequence number 35.

Preferred NASBA primers: H31e6704p1 and H31e6704p2
Preferred PCR primers: H31e6704PCR1 and H312e6704PCR2

(15) an oligonucleotide primer comprising sequence number 36 and an oligonucleotide primer comprising sequence number 37;

Preferred NASBA primers: H31e6705p1 and H31e6705p2
Preferred PCR primers: H31e6705PCR1 and H31e6705PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 33:

(16) an oligonucleotide primer comprising sequence number 38 and an oligonucleotide primer comprising sequence number 39; oligonucleotide probe comprising sequence number 40.

Preferred NASBA primers: H33e6701p1 and H33e6701p2
Preferred PCR primers: H33e6701PCR1 and H33e6701PCR2

(17) an oligonucleotide primer comprising sequence number 41 and an oligonucleotide primer comprising sequence number 42; oligonucleotide probe comprising sequence number 43.

Preferred NASBA primers: H33e6703p1 and H33e6703p2
Preferred PCR primers: H33e6703PCR1 and H33e6703PCR2

(18) an oligonucleotide primer comprising sequence number 44 and an oligonucleotide primer comprising sequence number 45; oligonucleotide probe comprising sequence number 46.

Preferred NASBA primers: H33e6702p1 and H33e6702p2
Preferred PCR primers: H33e6702PCR1 and H33e6702PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 35:

(19) an oligonucleotide primer comprising sequence number 47 and an oligonucleotide primer comprising sequence number 48; oligonucleotide probe comprising sequence number 53.

Preferred NASBA primers: H35e6701p1 and H35e6701p2
Preferred PCR primers: H35e6701PCR1 and H35e6701PCR2

(20) an oligonucleotide primer comprising sequence number 49 and an oligonucleotide primer comprising sequence number 50; oligonucleotide probe comprising sequence number 54.

Preferred NASBA primers: H35e6702p1 and H35e6702p2
Preferred PCR primers: H35e6702PCR1 and H35e6702PCR2

(21) an oligonucleotide primer comprising sequence number 51 and an oligonucleotide primer comprising sequence number 52; oligonucleotide probe comprising sequence number 55.

Preferred NASBA primers: H35e6703p1 and H35e6703p2
Preferred PCR primers: H35e6703PCR1 and H35e6703PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 52:

(22) an oligonucleotide primer comprising sequence number 56 and an oligonucleotide primer comprising sequence number 57; oligonucleotide probe comprising sequence number 58.

Preferred NASBA primers: H52e6701p1 and H52e6701p2
Preferred PCR primers: H52e6701PCR1 and H52e6701PCR2

(23) an oligonucleotide primer comprising sequence number 59 and an oligonucleotide primer comprising sequence number 60; oligonucleotide probe comprising sequence number 61.

Preferred NASBA primers: H52e6702p1 and H52e6702p2
Preferred PCR primers: H52e6702PCR1 and H52e6702PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 58:

(24) an oligonucleotide primer comprising sequence number 62 and an oligonucleotide primer comprising sequence number 63; oligonucleotide probe comprising sequence number 66.

Preferred NASBA primers: H58e6701p1 and H58e6701p2
Preferred PCR primers: H58e6701PCR1 and H58e6701PCR2

(25) an oligonucleotide primer comprising sequence number 64 and an oligonucleotide primer comprising sequence number 65; oligonucleotide probe comprising sequence number 67.

Preferred NASBA primers: H58e6702p1 and H58e6702p2
Preferred PCR primers: H58e6702PCR1 and H58e6702PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 51:

(26) an oligonucleotide primer comprising sequence number 104 and an oligonucleotide primer comprising sequence number 105; oligonucleotide probe comprising sequence number 108.

Preferred NASBA primers: H51e6701p1 and H51e6701p2
Preferred PCR primers: H51e6701PCR1 and H51e6701PCR2

(27) an oligonucleotide primer comprising sequence number 106 and an oligonucleotide primer comprising sequence number 107; oligonucleotide probe comprising sequence number 109.

Preferred NASBA primers: H51e6702p1 and H51e6702p2
Preferred PCR primers: H51e6702PCR1 and H51e6702PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 56:

(28) an oligonucleotide primer comprising sequence number 110 and an oligonucleotide primer comprising sequence number 111; oligonucleotide probe comprising sequence number 116.

Preferred NASBA primers: H56e6701p1 and H56e6701p2
Preferred PCR primers: H56e6701PCR1 and H56e6701PCR2

(29) an oligonucleotide primer comprising sequence number 112 and an oligonucleotide primer comprising sequence number 113; oligonucleotide probe comprising sequence number 117.

Preferred NASBA primers: H56e6702p1 and H56e6702p2
Preferred PCR primers: H56e6702PCR1 and H56e6702PCR2

(30) an oligonucleotide primer comprising sequence number 114 and an oligonucleotide primer comprising sequence number 115; oligonucleotide probe comprising sequence number 118 or sequence number 119.

Preferred NASBA primers: H56e6703p1 and H56e6703p2
Preferred PCR primers: H56e6703PCR1 and H56e6703PCR2

(31) an oligonucleotide primer comprising sequence number 120 and an oligonucleotide primer comprising sequence number 121; oligonucleotide probe comprising sequence number 122.

Preferred NASBA primers: H56e6704p1 and H56e6704p2
Preferred PCR primers: H56e6704PCR1 and H56e6704PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 39:

(32) an oligonucleotide primer comprising sequence number 80 and an oligonucleotide primer comprising sequence number 81; oligonucleotide probe comprising sequence number 82.

Preferred NASBA primers: H39e6701p1 and H39e6701p2
Preferred PCR primers: H39e6701PCR1 and H39e6701PCR2

(33) an oligonucleotide primer comprising sequence number 83 and an oligonucleotide primer comprising sequence number 84; oligonucleotide probe comprising sequence number 85.

Preferred NASBA primers: H39e6702p1 and H39e6702p2
Preferred PCR primers: H39e6702PCR1 and H39e6702PCR2

(34) an oligonucleotide primer comprising sequence number 86 and an oligonucleotide primer comprising

sequence number 87; oligonucleotide probe comprising sequence number 88.

Preferred NASBA primers: H39e6703p1 and H39e6703p2
Preferred PCR primers: H39e6703PCR1 and H39e6703PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of HPV 45:

(35) an oligonucleotide primer comprising sequence number 89 and an oligonucleotide primer comprising sequence number 90; oligonucleotide probe comprising sequence number 93.

Preferred NASBA primers: H45e6701p1 and H45e6701p2
Preferred PCR primers: H45e6701PCR1 and H45e6701PCR2

(36) an oligonucleotide primer comprising sequence number 91 and an oligonucleotide primer comprising sequence number 92; oligonucleotide probe comprising sequence number 94.

Preferred NASBA primers: H45e6702p1 and H45e6702p2
Preferred PCR primers: H45e6702PCR1 and H45e6702PCR2

(37) an oligonucleotide primer comprising sequence number 95 and an oligonucleotide primer comprising sequence number 96; oligonucleotide probe comprising sequence number 101.

Preferred NASBA primers: H45e6703p1 and H45e6703p2
Preferred PCR primers: H45e6703PCR1 and H45e6703PCR2

(38) an oligonucleotide primer comprising sequence number 97 and an oligonucleotide primer comprising sequence number 98; oligonucleotide probe comprising sequence number 102.

Preferred NASBA primers: H45e6704p1 and H45e6704p2
Preferred PCR primers: H45e6704PCR1 and H45e6704PCR2

(39) an oligonucleotide primer comprising sequence number 99 and an oligonucleotide primer comprising sequence number 100; oligonucleotide probe comprising sequence number 103.

Preferred NASBA primers: H45e6705p1 and H45e6705p2
Preferred PCR primers: H45e6705PCR1 and H45e6705PCR2

Primer-pairs for use in the detection of mRNA transcripts from the E6 gene of group B HPV:

(40) an oligonucleotide primer comprising sequence number 68 and an oligonucleotide primer comprising sequence number 69; oligonucleotide probe comprising sequence number 72.

Preferred NASBA primers: HBe6701p1 and HBe6701p2
Preferred PCR primers: HBe6701PCR1 and HBe6701PCR2

(41) an oligonucleotide primer comprising sequence number 70 and an oligonucleotide primer comprising sequence number 71; oligonucleotide probe comprising sequence number 73.

Preferred NASBA primers: HBe6702p1 and HBe6702p2
Preferred PCR primers: HBe6702PCR1 and HBe6702PCR2

(42) an oligonucleotide primer comprising sequence number 74 and an oligonucleotide primer comprising sequence number 75; oligonucleotide probe comprising sequence number 76.

Preferred NASBA primers: HBe6703p1 and HBe6703p2
Preferred PCR primers: HBe6703PCR1 and HBe6703PCR2

Primer-pair for use in the detection of mRNA transcripts from the E6 gene of group C HPV:

(43) an oligonucleotide primer comprising sequence number 77 and an oligonucleotide primer comprising sequence number 78; oligonucleotide probe comprising sequence number 79.

Preferred NASBA primers: HCe6701p1 and HCe6701p2
Preferred PCR primers: HCe6701PCR1 and HCe6701PCR2

Methods of detecting HPV

In a further aspect the invention provides a method for detecting HPV mRNA in a test sample suspected of containing HPV which comprises performing an amplification reaction on the test sample to amplify a portion of the mRNA transcribed from the E6 gene of HPV, wherein the amplification reaction is performed using one of the primer-pairs provided by the invention, as defined above.

Preferred amplification techniques which may be used to amplify a portion of the E6 mRNA are RT-PCR or NASBA.

The "test sample suspected of containing HPV" will most commonly be a clinical sample, for example a cervical scraping in the cervical screening field. The amplification reaction will preferably be carried out on a preparation of nucleic acid isolated from the test sample. The preparation of nucleic acid must include mRNA, however it need not be a preparation of purified poly A+ mRNA and preparations of total RNA or crude preparations of total nucleic acid containing both RNA and genomic DNA are also suitable as starting material for a NASBA reaction. Essentially any technique known in the art for the isolation of a

preparation of nucleic acid including mRNA may be used to isolate nucleic acid from the test sample. A preferred technique is the "Boom" isolation method described in US-A-5,234,809 and EP-B-0389,063. This method, which can be used to isolate a nucleic acid preparation containing both RNA and DNA, is based on the nucleic acid binding properties of silicon dioxide particles in the presence of the chaotropic agent guanidine thiocyanate (GuSCN).

Methods for the detection of HPV in a test sample using the NASBA technique will generally comprise the following steps:

(a) assembling a reaction medium comprising a primer-pair according to the invention, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and deoxyribonucleoside triphosphates;

(b) incubating said reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

Detection of the specific product(s) of the NASBA reaction (i.e. sense and/or antisense copies of the target RNA) may be carried out in a number of different ways. In one approach the NASBA product(s) may be detected with the use of an HPV-specific hybridisation probe capable of specifically annealing to the NASBA product. The hybridisation probe may be attached to a revealing label, for example a

fluorescent, luminescent, radioactive or chemiluminescent compound or an enzyme label or any other type of label known to those of ordinary skill in the art. The precise nature of the label is not critical, but it should be capable of producing a signal detectable by external means, either by itself or in conjunction with one or more additional substances (e.g. the substrate for an enzyme).

Also within the scope of the invention is so-called "real-time NASBA" which allows continuous monitoring of the formation of the product of the NASBA reaction over the course of the reaction. In a preferred embodiment this may be achieved using a "molecular beacons" probe comprising an HPV-specific sequence capable of annealing to the NASBA product, a stem-duplex forming oligonucleotide sequence and a pair of fluorescer/quencher moieties, as known in the art described herein. If the molecular beacons probe is added to the reaction mixture prior to amplification it may be possible to monitor the formation of the NASBA product in real-time (Leone *et al.*, Nucleic Acids Research, 1998, Vol 26, 2150-2155).

In a further approach, the molecular beacons technology may be incorporated into the primer 2 oligonucleotide allowing real-time monitoring of the NASBA reaction without the need for a separate hybridisation probe.

In a still further approach the products of the NASBA reaction may be monitored using a generic labelled detection probe which hybridises to a nucleotide sequence in the 5' terminus of the primer 2 oligonucleotide. This is equivalent to the "NucliSens™" detection system supplied by Organon Teknika. In this system specificity for NASBA

products derived from the target HPV mRNA may be conferred by using HPV-specific capture probes comprising probe oligonucleotides as described herein attached to a solid support such as a magnetic microbead. Most preferably the generic labelled detection probe is the ECL™ detection probe supplied by Organon Teknika. NASBA amplicons are hybridized to the HPV-specific capture probes and the generic ECL probe (via a complementary sequence on primer 2). Following hybridization the bead/amplicon/ECL probe complexes may be captured at the magnet electrode of an automatic ECL reader (e.g. the NucliSens™ reader supplied by Organon Teknika. Subsequently, a voltage pulse triggers the ECL™ reaction.

Also provided by the invention are reagent kits for use in the detection of HPV by NASBA, the kits comprising a primer-pair cocktail according to the invention. The reagent kits may further comprise a mixture of enzymes required for the NASBA reaction, specifically an enzyme mixture containing an RNA directed DNA polymerase (e.g. a reverse transcriptase), a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA (e.g. RNaseH) and an RNA polymerase. The RNA polymerase should be one which recognises the promoter sequence present in the 5' terminal region of the NASBA P1 primer oligonucleotides in the oligonucleotide primer sets supplied in the reagent kit. The kit may also comprise a supply of NASBA buffer containing the ribonucleosides and deoxyribonucleosides required for RNA and DNA synthesis. The composition of a standard NASBA reaction buffer will be well known to those skilled in the art.

In certain embodiments the kit may further contain one or more capture probes, comprising a probe oligonucleotide attached to a solid support as described above, for immobilising the products of a specific NASBA reaction. The kit may still further contain labelled generic detection probes. Advantageously, the detection probes may comprise a sequence of nucleotides complementary to a non-HPV sequence present at the 5' terminal end of the NASBA P2 primer oligonucleotides present in the reagent kit.

In still further embodiments the kit may further contain one or more molecular beacon probes according to the invention. The molecular beacon probes may be supplied as a separate reagent within the kit. Alternatively, the NASBA primers and molecular beacons probe may be supplied as a primer/probe mixture. Such a mixture including the NASBA P1 and P2 primers and also a molecular beacons probe is convenient for use in "real-time" NASBA, wherein the NASBA amplification reaction and detection of an amplification product are performed simultaneously in a single reaction vessel.

The invention will be further understood with reference to the following, non-limiting, Example:

Example 1-Real-time NASBA

Collection and preparation of clinical samples

Cervical cytobrush samples are collected in 9 ml lysis buffer (5M Guanidine thiocyanate) prior to RNA/DNA extraction. Since RNA is best protected in the 5M guanidine thiocyanate at -70°C only 1 ml of the total volume of sample is used for each extraction round. 2-3 tubes with the RNA/DNA are stored at -167°C and the rest stored at -70°C.

RNA and DNA were automatically isolated according to the "Booms" isolation method from Organon Teknika (Organon Teknika B.V., Boselind 15, P.O. Box 84, 5280 AB Baxtel, The Netherlands; now Biomérieux, 69280 Marcy l'Etoile, France).

The following procedure was carried out using reagents from the Nuclisens™ Basic Kit, supplied by Organon Teknika. Procedure for n=10 samples:-

1. Prepare enzyme solution.
Add 55 µl of enzyme diluent (from Nuclisens™ Basic Kit; contains sorbitol in aqueous solution) to each of 3 lyophilized enzyme spheres (from Nuclisens™ Basic Kit; contains AMV-RT, RNase H, T7 RNA polymerase and BSA). Leave this enzyme solution at least for 20 minutes at room temperature. Gather the enzyme solutions in one tube, mix well by flicking the tube with your finger, spin down briefly and use within 1 hour. Final concentrations in the enzyme mix are 375 mM sorbitol, 2.5 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase.

2. Prepare reagent sphere/KCl solution.
For 10 samples: add 80 µl reagent sphere diluent (from Nuclisens™ Basic Kit; contains Tris/HCl (pH 8.5), 45% DMSO) to the lyophilized reagent sphere (from Nuclisens™ Basic Kit; contains nucleotides, dithiotreitol and $MgCl_2$) and immediately vortex well. Do this with 3 reagent spheres and mix the solutions in one tube.

Add 3 µl NASBA water (from Nuclisens™ Basic Kit) to the reconstituted reagent sphere solution and mix well.

Add 56 µl of KCl stock solution (from Nuclisens™ Basic Kit) and mix well. Use of this KCl/water mixture will result in NASBA reactions with a final KCl concentration of 70 mM. Final concentrations in the reagent/KCl solution are 1 mM of each dNTP, 2 mM of ATP, UTP and CTP, 1.5 mM GTP, and 0.5 mM ITP, 0.5 mM dithiotreitol, 70 mM KCl, 12 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.5).

3. Prepare primer/probe solution containing target-specific primers and molecular beacon probe.
For each target reaction transfer 91 µl of the reagent sphere/KCl solution (prepared in step 2) into a fresh tube. Add 25 µl of primers/molecular beacon probe solution (to give final concentration of ~0.1-0.5 µM each of the sense and antisense primers and ~ 15-70 pmol molecular beacon probe per reaction). Mix well by vortexing. Do not centrifuge.

In case less than 10 target RNA amplifications are being performed refer to the table below for the appropriate amounts of reagent sphere solution, KCl/water solution and primers to be used. Primer solutions should be used within 30 minutes after preparation.

Reactions (n)	Reagent sphere solution (µl)	KCl/water (µl)	Primer mix (µl)
10	80	30	10
9	72	27	9
8	64	24	8
7	56	21	7
6	48	18	6
5	40	15	5
4	32	12	4
3	24	9	3
2	16	6	2
1	8	3	1

4. Addition of samples

For each target RNA reaction:

In a 96 well microtiter plate pipette 10 µl of the primer/probe solution (prepared in step 3) into each of 10 wells. Add 5 µl nucleic acid extract to each well. Incubate the microtiter plate for 4 minutes at 65 ± 1 °C. Cool to at 41 ± 0.5 °C for 4 minutes. Then to each well add 5 µl enzyme solution. Immediately place the microtiter plate in a fluorescent detection instrument (e.g. NucliSens™ EasyQ Analyzer) and start the amplification.